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Laurent Nevarez, Valérie Vasseur*, Stella Debaets, Georges Barbier

-Université Européenne de Bretagne, France

-Université de Brest, EA3882 Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, IFR148 ScInBioS, ESMISAB, Technopôle de Brest Iroise, 29280 Plouzané, France

* Corresponding author. Phone: +33 2 98 05 61 00; Fax: +33 2 98 05 61 01

E-mail address: valerie.vasseur@univ-brest.fr

ABSTRACT

Fungi are ubiquitous micro-organisms often associated with spoilage and biodeterioration of a large variety of foods and feedstuffs. Their growth may be influenced by temporary changes in intrinsic or environmental factors such as temperature, water activity, pH, preservatives, atmosphere composition, all of which may represent potential sources of stress. Molecular-based analyses of their physiological responses to environmental conditions would help to better manage the risk of alteration and potential toxicity of food products. However, before investigating molecular stress responses, appropriate experimental stress conditions must be precisely defined. *Penicillium glabrum* is a filamentous fungus widely present in the environment and frequently isolated in the food processing industry as a contaminant of numerous products. Using response surface methodology, the present study evaluated the influence of two environmental factors (temperature and pH) on *P. glabrum* growth to determine 'optimised' environmental stress conditions. For thermal and pH shocks, a large range of conditions was applied by varying factor intensity and exposure time according to a two-factorial central composite design. Temperature and exposure duration varied from 30 to 50°C and from 10 min to 230 min, respectively. The effects of interaction between both variables were observed on fungal growth. For pH, the duration of exposure, from 10 to 230 min, had no significant effect on fungal growth. Experiments were thus carried out on a range of pH from 0.15 to 12.50 for a single exposure time of 240 min. Based on fungal growth results, a thermal shock of 120 min at 40°C or a pH shock of 240 min at 1.50 or 9.00 may therefore be useful to investigate stress responses to non-optimal conditions.

Keywords: Penicillium glabrum, food spoilage, thermal shock, acid shock, alkali shock, mycelial growth, response surface methodology, central composite design.

Introduction

Fungi are ubiquitous micro-organisms often associated with spoilage and biodeterioration of a large variety of foods and feedstuffs. Various genera such as *Aspergillus*, *Cladosporium*, *Alternaria* or *Penicillium* are involved in different food spoilage (Pitt & Hocking 1997). *Penicillium* is one of the most widespread fungal genera isolated from food products and, in addition to the economic losses they cause, several *Penicillium* species may produce mycotoxins that represent a potential health risk for humans and animals (Pitt & Hocking 1997; Samson et al. 2004). Numerous intrinsic parameters in foods (water activity, pH, preservatives, *etc.*) or extrinsic ones (temperature, atmosphere composition, *etc.*) provide favourable conditions for moulds to develop. Changes in these parameters beyond the tolerated range may represent a potential source of stress that can affect germination, mycelial growth, conidiation or even synthesis of secondary metabolites as mycotoxins (Espeso et al. 1993; Calvo et al. 2002; Magan et al. 2002; Schmidt-Heydt et al. 2008).

Nevertheless, to our knowledge, little is known about the effects of shock conditions on the growth and physiology of food related filamentous fungi. Understanding these effects in spoilage moulds would be useful for a better risk management of alteration and toxicity of food products. In this study, investigations were conducted on *Penicillium glabrum*, which is very frequently encountered in the food processing industry due to its ubiquitous presence in the environment and its capacity to disperse a large number of spores into the air (Pitt & Hocking 1997). This fungal contaminant was has been isolated in a large variety of products, including cheese (Hocking & Faedo 1992), nuts (Freire et al. 2000), bottled mineral water (Cabral & Fernandez Pinto 2002), *etc*. In our study, the effects of thermal and pH shock conditions were investigated in *P. glabrum* grown in liquid cultures. Temperature was chosen for this study because it is one of the most important factors that determine the ability of moulds to grow (Dantigny et al. 2005). pH was also investigated as this is a main environmental factor of physiological importance that can vary significantly depending on the food product. Shock conditions are usually defined by their intensity and their duration. To efficiently study the physiological effect of a large range of these two environmental shock conditions, we analysed *P. glabrum* growth using the response surface methodology (RSM) (Myers et al. 1989). A two-factorial central composite design (CCD) (Box et al. 1978) was applied to determine 'optimised' experimental shock conditions by combining different stress intensities and durations for each factor studied. Results from CCD were used to modelise the effects of shock intensity and duration on the measured response (fungal biomass growth).

This study brings a missing characterisation of the growth response of a fungal food contaminant to different thermal, acidic and alkaline conditions. Such results were needed to support the choice of temperature and pH shock conditions to investigate stress response of *P. glabrum*.

Materials and methods

Fungal strain and culture medium

The fungal strain used in this study was isolated from contaminated, aromatised mineral water. It was characterised as *Penicillium glabrum* (Wehmer) Westling according to the reference method for classifying *Penicillium* species based on morphological characteristics (Pitt, 1988). To confirm the morphological identification of the fungal strain, we sequenced the internal transcribed spacer region including the 5.8S of the ribosomal gene in both directions after PCR amplification. Species identification was confirmed based on the results of BLAST (Basic Local Alignments Search Tool) searches against known sequences in the GenBank database using BLASTn. The strain has been registered as LMSA 1.01.421 in the Brittany Microbe Culture Collection (*Souchothèque de Bretagne*; University of Brest, France; www.ifremer.fr/souchotheque) and as LCP 08.5568 in the fungal collection of the Laboratory of Cryptogamy at the *Museum National d'Histoire Naturelle* (Paris, France; www.mnhn.fr). The cardinal (minimum, optimum and maximum) growth conditions of temperature and pH for this filamentous fungus have been estimated to be 6.6 °C, 24.3 °C and 33.8 °C and pH 0.50 - 1.00, pH 5.50 and pH 11.20 (Nevarez et al. 2009).

For strain maintenance and collection of spores, the strain was cultured in tubes of potato dextrose agar medium (PDA, Difco Laboratories, Detroit, MI, USA) at 25 °C. The pH of this culture medium was 5.00.

Shock application on *P. glabrum* liquid cultures

Spores were collected from seven-day-old mycelium by flooding each tube with 2 ml of sterile water containing 0.01 % of Tween 80 (Sigma-Aldrich, Saint Louis, MO, USA) under agitation at 250 rpm. Mycelia were cultured from a suspension of 5×10^6 *P. glabrum* spores inoculated into a 250 ml Erlenmeyer flask containing 50 ml of potato dextrose broth medium (PDB, Difco Laboratories). Cultures of *P. glabrum* were first grown in standard PDB (pH = 5.0) at 25 °C, 120 rpm for 48 h and then subjected to different experimental conditions. To test response to a range of thermal conditions, mycelia were aseptically filtered at room temperature and transferred immediately into 50 ml of standard PDB media and incubated at different temperature conditions at 120 rpm. For efficient incubation, each inoculated medium had been previously warmed to its corresponding experimental temperature. To test response to pH conditions, mycelia grown for 48 h were aseptically filtered and transferred into modified PDB medium at different pH values. Each medium was prepared with PDB and to stabilise the pH, appropriate buffers were added: citrate-phosphate buffer for pH from 2.00 to 8.00, borate buffer for pH from 8.00 to 9.50, CAPS (Ncyclohexyl-3-aminopropanesulfonic acid) buffer for pH 9.50 from to 11.50. For marginal pH conditions above pH 11.50 or below pH 2.00, buffer was not required for pH stability. The pH was then adjusted with NaOH and HCl (1, 2 or 10 M). The pH of each adjusted PDB was verified. The media were filtered through a 0.22 µm membrane and 50 ml was aseptically distributed into sterile 250 ml Erlenmeyer flasks.

The pH of each adjusted medium was assessed after fungal culture and variation observed in each medium before inoculation was less than 0.5 pH units. These results confirmed the efficiency of the various buffers used to prepare the adjusted media.

After incubation under thermal or pH experimental conditions, the mycelia were aseptically filtered at room temperature and transferred into standard PDB medium (pH 5.00) for cultivation in optimal conditions for 48 h at 25 °C, 120 rpm. Mycelia were then filtered, washed thoroughly with distilled water, dried at 70 °C for 48 h and weighed with a precision balance. Mycelium growth was obtained by quantifying the mycelium dry mass.

Other than the experimental cultures of *P. glabrum* for each tested environmental factor (temperature, pH), two sets of control 'unstressed' mycelia were also cultured under optimum conditions at 25 °C. The first control, T48, was early-harvested after 48 h of culture and the dry biomass obtained was used as the minimum fungal growth reached by each culture before application of experimental thermal and pH conditions. A second control, T96, was harvested after 96 h culture and corresponded to 48 h of culture under optimum conditions in standard PDB at 25 °C and then aseptically filtered and transfered to standard PDB at 25°C for another 48 h. The dry fungal biomass obtained for this control was considered as the fungal growth after 96 h culture in optimum conditions. The growth results obtained for each temperature or pH condition were expressed as the relative growth rate, in percent. This is the ratio between the fungal dry weight obtained under a given experimental condition and the fungal dry weight obtained in the control T96.

Experimental design and statistical analysis

The thermal and pH stress experiments were conducted according to a two-factorial central composite design (Box et al. 1978) (Fig 1). For each of the three applied stresses (thermal, acid and alkali stress), the CCD (Factors/Blocks/Runs = 2/1/11) defined nine experimental conditions by varying the intensity of the tested environmental factor and its duration (Table 1). Given the biological variability of *P. glabrum* growth, each experimental point was replicated three times to increase precision (consequently, central conditions of each CCD were replicated nine times) and median values of those three replicates were considered. The growth results obtained for thermal, acid or alkali stress experiments were analysed separately using STATISTICA 8 (StatSoft) and a response surface was determined to model the effect of different stress on *P. glabrum* growth. The growth results of both controls (T48 and T96) were also reported in the response surface for comparison with CCD results.

For pH investigations, another experimental design was also implemented. Twenty pH values were studied in a large range from 0.15 to 12.50 with a single duration of 240 min. To obtain better estimates, four replicates were studied for each experimental condition. The results obtained were analysed with STATGRAPHICS 5.0 (Statistical Graphics Corp) using a one-way ANOVA and the LSD test to determine which fungal growths were significantly different depending on the tested pH condition. For each condition, means, (which were very close to median values) were plotted and LSD results were reported by assigning letters to the means. Means with the same letter are not statistically different ($P > 0.05$).

Results

Effect of thermal shock on fungal growth

To investigate the effect of temperature on *Penicillium glabrum* growth, two-factorial CCD and RSM were used. Temperature and exposure duration varied respectively from 30 to 50 °C and from 10 min to 230 min. The low, middle and high levels of both variables were determined from preliminary experiments on fungal growth (unpublished data).

P. glabrum growth results obtained using a CCD were statistically analysed and both parameters (temperature level and exposure time) and their interaction had significant effects ($P < 0.05$) on fungal growth. A response surface was determined from the results (Fig 2) and the goodness-of-fit between the predicted values and the experimental data was very high (Fig 3), giving a coefficient of determination (R^2) of 0.98, indicating a very good adjustment of the model with experimental data.

Inspection of the response surface showed that increasing temperature and exposure time affect *P. glabrum* growth in a gradual manner.

The adjusted surface response (Fig 2), showed three distinct response areas delimited by the results of both growth controls T96 and T48 (which was 48 % of T96). The first response area was observed for the less intense thermal shock conditions 30 °C:120 min to 40 °C:10 min and revealed an increase of fungal growth in comparison with the fungal biomass of the control T96. A clear fungal growth increase was measured for the lowest shock condition (30°C:120 min) at supra-optimal temperature (Nevarez et al. 2009). A second area was observed for intermediate thermal conditions (40°C:230 min, 40 °C:120 min or 47°C:42 min), which showed a moderate reduction in fungal biomass compared to the T96 control but still greater than the fungal growth of the T48 control. The third area was observed for the highest thermal conditions, e.g. 47°C:198 min or 50 °C:120 min, and revealed a strong decrease in *P. glabrum* growth. Fungal biomass values were even lower than those of the T48 control.

Considering the fungal growth results, thermal conditions as 40 °C:120 min, 40 °C:230 min or 47 °C:42 min, appeared to affect moderately *P. glabrum* growth.

Effect of pH shock on fungal growth

Statistical analysis of the results obtained using a CCD for acid and alkali pH indicated that exposure time had no significant effect ($P>0.05$) on fungal growth for the range of values tested (10 to 230 min) (data not shown). This result precluded 2D analysis and response surface modelling, since only one of the two tested variables (pH value) had a significant influence. Consequently, we modified our experimental design by using a monofactorial experimental procedure on a large range of pH from 0.15 to 12.50 for a single exposure time of 240 min.

Global inspection of the results (Fig 4) shows that *P. glabrum* is able to grow relatively well after 240 min of exposure in a very wide range of pH conditions, spanning pH 2.00 to 11.50.

Analysis of fungal growth clearly showed four distinct areas of response over the wide range of pH conditions tested. The first area was observed for pH 2.00 to 7.00, which had a very low influence on fungal growth (80-100 % of T96 growth). The second area was observed for alkaline conditions, from pH 8.00 to 11.50, which induced a decrease in fungal growth, showing a growth rate of 60-70 % of T96 growth.

The third and fourth response areas were observed for very acidic conditions (pH 0.15 to 1.00) or alkaline conditions (pH 12.00 to 12.50). These conditions induced a dramatic decrease in fungal growth after 240 min of exposition. The fungal biomass values were even lower than the control T48 value.

Considering the fungal growth results obtained, alkaline shock conditions between pH 8.00 to 11.50 for 240 min duration affect significantly and moderately *P. glabrum* development. Concerning acidic shock, the transition from no detrimental to detrimental effect appears very steep.

Discussion

To investigate the effect of temperature and pH shocks on *Penicillium glabrum* growth, two-factorial CCD and RSM were employed. Using a CCD offers the possibility to assess the effect of a large range of conditions by testing a limited and optimised number of experimental points with a low number of replicates. This approach has been successfully employed in fungi to examine chitinase regulation (Lopes et al. 2008), to study the influence of environmental factors such as temperature and pH on the growth of the fermenting yeast *Pachysolen tannophilus* used in industrial fermentation processes (Roebuck et al. 1995) or to investigate the influence of temperature, pH and a_w on yeast to study their antagonistic properties (Sinigaglia et al. 1998). RSM is a well-known method for optimising for example medium composition or other critical variables that affect for example enzyme production or microbial growth. This study used RSM to predict fungal growth anywhere within the limits of the experimentally tested environmental factors. The quadratic model obtained in those conditions permitted suitable predictions.

Regarding temperature, inspection of the response surface clearly shows that increasing temperature and exposure duration affect *P. glabrum* growth in a gradual manner. The influence of a given temperature in a wide range of exposure duration has been also observed in *Saccharomyces cerevisiae* exposed to 37 °C for 15, 30, 45, 60, 120, 240 and 480 min (Sakaki et al. 2003).

The less intense shock condition (30°C:120 min) did not reduce *P. glabrum* fungal growth, but showed an unexpected increase in fungal biomass. This result suggests that when a fungal contaminant such as *P. glabrum* is exposed to relatively weak stress conditions, its development on food products may be enhanced. This observation could be practically important for food industry.

The intermediate stress conditions (40°C:120 min, 40°C:230 min, 47°C:42 min) induced more or less moderate fungal biomass reductions which can be attributed simultaneously to growth reduction or increased lag time for growth due to thermal shock. This fungal growth reduction could be explained by a variety of cellular effects corresponding with thermal stress. It is known that elevated temperature can affect: (i) the structure of proteins, possibly modifying their biological activity and overall cellular functioning; (ii) the biosynthesis of a large number of ubiquitous proteins which decrease or are completely stopped (Plesofsky-Vig & Brambl 1987; Curle & Kapoor 1988); and (iii) plasma membrane fluidity (Beney & Gervais 2001). As described in *S. cerevisiae*, an increase of plasma membrane permeability can affect cellular integrity and metabolism (Guyot et al. 2005).

Finally, in more drastic thermal conditions (47°C:198 min, 50°C:120 min), the fungal biomass decreased compared to the initial fungal biomass (T48) before applying the experimental stress conditions. According to the literature, this decrease can be explained by fungal lysis (Emri et al. 2004; Koutinas et al. 2005) or may be due to an ordered degradation of cellular reserves (McNeil et al. 1998). In yeast and filamentous fungi, autolysis can be characterised by a fungal biomass decline. Autolysis occurs in response to a wide range of extrinsic factors such as heat, chemical treatment, nutrient starvation, *etc*., which may induce the loss of membrane function leading to a breakdown in intracellular compartimentalisation and the release of lytic enzymes responsible of macromolecular degradation (Hernawan & Fleet 1995; McNeil et al. 1998). Important reduction in fungal growth under high temperatures such as 50 °C has also been reported in *S. cerevisiae* (Seppa et al. 2004) and *Neurospora crassa* (Plesofsky-Vig & Brambl 1985), for which optimum growth conditions are 25°C and 30°C, respectively. The yeast *Candida albicans*, for which optimum growth temperature is 37°C, is also very affected by exposure at 55 °C (Zeuthen & Howard 1989). In our case, thermal conditions of 47°C:198 min or 50°C:120 min appeared to affect not only *P. glabrum* fungal growth but also proteins and mRNA integrity. In fact, subjecting total proteins to electrophoresis showed that most of the proteins observed in the T96 control condition were not visible in the highest stress conditions mentioned above (data not shown). Moreover, we analysed the total RNA using electrophoretic RNA separation on microfabricated chips to determine their quality. In the drastic growth conditions, this analysis revealed a decrease in the 18S and 28S ribosomal RNA peaks and an increase in smaller, intermediate RNA fragments, indicating substantial total RNA degradation (Nevarez et al. 2008).

Given the results on fungal growth, thermal conditions such as 40°C:120 min, 40°C:230 min or 47°C:42 min, appeared to moderately, but significantly affect *P. glabrum* growth. These conditions may therefore be appropriate for further investigations on stress response. In addition, these thermal shock conditions (temperature level and exposure time) correspond pretty well with those used in other thermal stress studies conducted in fungi which are unfrequently justified. For example, a study was realised in *S. cerevisiae* in order to isolate heat shock proteins (HSP), HSP 82 and HSP 104 by shifting optimal cultures from 25 °C to 39 °C for 1 h (Sanchez et al. 1993). Other investigations conducted in *N. crassa* or *C. albicans* exposed fungi to experimental conditions from 30 °C to 45 °C for 90 min and from 37 °C to 40 - 46 °C for 30 min, respectively (Plesofsky-Vig & Brambl 1985; Zeuthen & Howard 1989). Thermal stress was also investigated with a transcriptional approach using microarrays in yeasts *S. cerevisiae* or *Schizosaccharomyces pombe*, from 25°C to 37°C for 2 h or 30°C to 39°C for 1 h, respectively (Causton et al. 2001; Chen et al. 2003). In our case, where physiological experimental stress conditions were being validated, the thermal condition of 40°C:120 min was employed as central condition to investigate heat shock response at the transcriptional level (Nevarez et al. 2008). A transcriptional study combining suppression-subtraction hybridisation and cDNA microarrays has been conducted in this fungus to isolate differentially expressed genes in response to thermal shock. Of the various isolated genes, a few are down-regulated and encode for proteins involved in general cellular metabolism. Given this thermal shock condition, gene down-regulation may explain the *P. glabrum* growth reduction as observed in this study.

The effect of a very large range of pH conditions was tested on *P. glabrum* growth for a single exposure time of 240 min. High pH tolerance has been already described in many filamentous fungi which appear to be little affected by changes in culture pH (Wheeler et al. 1991). Many *Penicillium* species including *P. chrysogenum*, *P. camemberti*, *P. aurantiogriseum*, *P. marneffei*, *P. crustosum* or *P. islandicum* are able to grow from pH 3.00 - 4.00 to 9.00 - 10.00 (Wheeler et al. 1991; Thompson et al. 1993; Cao et al. 2007). The ability of fungi to develop in a wide range of pH is partially due to adaptation associated with a genetic regulatory system that tailors gene expression to the ambient pH (Arst & Penalva 2003). Considerable progress has been made in characterising fungal genetic pH regulatory systems because they are important for major processes including pathogenesis and the production of extracellular enzymes, penicillin or mycotoxins (Espeso et al. 1993; Denison 2000). Various pH regulatory systems have been described in fungi (*Aspergillus nidulans*, *A. niger*, *Penicillium chrysogenum*, *S. cerevisiae*, *C. albicans*, *Yarrowia lipolytica*; Denison 2000; Arst & Penalva 2003).

In acidic conditions (pH 2.00 to 7.00), fungal growth was only slightly affected. This observation is in accordance with reports that most filamentous fungi including *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp. show high tolerance to acidic media and their optimum growth is around pH 5.50 - 6.00 (Deacon 2006). Similar observations have been made on several *Penicillium* species such as *P. citreonigrum*, *P. jensenii* or *P. roqueforti* (Sacks et al. 1986; Wheeler et al. 1991; Gock et al. 2003). It has also been shown in *S. cerevisiae* that yeast cells grow more rapidly in acidic media than in neutral or alkaline media (Lamb et al. 2001). An important factor for the maintenance of an acidic environment is the yeast plasma membrane H^+-ATPase, which actively extrudes protons and imports many nutrients and cations (Serrano et al. 2002).

The alkaline pH conditions (pH 8.00 to 11.50) induced a greater growth decrease area than observed in acidic conditions. This effect may be explained by some cellular modifications caused by alkaline pH. In *S. cerevisiae* for example, it has been shown that alkaline media induce disruption of membrane proton gradients that normally supply energy for nutrient and ion transport essential for fungal development (Lamb et al. 2001). Some authors also report in *S. cerevisiae* a significant repression of genes involved in amino acid or purine biosynthesis and in carbohydrate metabolism that could explain limitation of yeast growth (Serrano et al. 2002). These authors suggest that copper or iron availability and solubility can be reduced by alkaline pH, which could affect some enzymatic activities. Thus, highly alkaline environments can be considered as stressing conditions.

Given the results on fungal growth, alkaline stress conditions between pH 8.00 to 11.50 for 240 min may be appropriate for further investigations on stress response as they seemed to moderately, but significantly affect fungal development. On the basis of our results, the experimental point pH 9.00:240 min may be employed as central condition for further studies in *P. glabrum.* Comparable alkaline pH conditions have also been used in several studies on fungi as, for example, in *C. glabrata* to analyse its pH response by transferring cultures from pH 4.00 to pH 8.00 (Schmidt et al. 2008). Some molecular studies have been also conducted at pH 4.00 to 8.00 in *C. albicans* or *A.*

nidulans to investigate the role of pH transcription factors (Rim13p and PacC respectively) (Espeso & Arst 2000; Li et al. 2004). A transcriptional approach using microarrays has been employed in *S. cerevisiae* at pH ranging from 6.00 to 7.90 (Causton et al. 2001).

Finally, in extreme pH conditions (pH 0.15 to 1.00 and pH 12.00 to 12.50), a great decrease in fungal biomass was induced. These drastic conditions severely affected cellular metabolism. As suggested for thermal stress, the effect induced by these conditions on *P. glabrum* growth may be also explained by fungal autolysis (McNeil et al. 1998).

In summary, the present work investigated a wide range of temperature and pH conditions to analyse their effect on *P. glabrum* growth to determine 'optimised' experimental shock conditions. To our knowledge, this approach has not been previously reported in other fungal stress studies, which generally use a limited number of experimental conditions. The results obtained here made it possible to determine experimental conditions that may be potentially appropriate for further investigations on stress response. Based on our results, we propose that conditions such as 40°C:120 min, pH 1.50:240 min or pH 9.00:240 min, can be used to produce a physiological stress response because they moderately, but significantly affect growth in *P. glabrum*.

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